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# Differential characteristics of salicylic acid-mediated signaling in potato

D.A. Navarre[a,b,*], D. Mayo[a]

[a]*USDA-Agricultural Research Service, 24106 N. Bunn Rd, Prosser, WA 99350, USA*
[b]*Department of Plant Pathology, Washington State University, 24106 N. Bunn Rd, Prosser, WA 99350, USA*

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**Abstract**

Potato (*Solanum tuberosum*) has key differences in salicylic acid (SA) metabolism and signaling from tobacco and *Arabidopsis* such as high basal salicylic acid concentrations in all tissues examined, including roots and tubers. Potato has high levels of basal *PR-1* expression and this appears to be a consequence of the high SA levels, because the expression is reduced in *nahG* plants. Despite the high basal SA levels, potato was nevertheless responsive to exogenous SA and as little as 250 μM SA-induced *PR-1* when sprayed on plants. However, plants grown in field conditions appeared less responsive to BTH at certain times. In spite of the high total SA concentrations, some potato varieties are able to maintain free SA levels as low as 0.5% of the total SA. Potato is also unusual in that *PR-1* is strongly induced in leaf discs in the absence of any additional treatment. Furthermore, potato leaf discs are hypersensitive to BTH, with concentrations as low as 1 μM causing extensive cell death, whereas concentrations as high as 500 μM had no such effect on tobacco.
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*Keywords:* Defense signaling; Disease resistance

## 1. Introduction

Inducible plant defenses such as systemic acquired resistance (SAR) are a key component of a plant's repertoire of disease resistance mechanisms and are a promising target to manipulate for improved disease control. During SAR, plants successfully resisting a pathogen can become highly resistant to subsequent infection not only by the original pathogen, but a variety of other pathogens [5,10,22]. Induced resistance pathways are regulated by key signal molecules like salicylic acid (SA), jasmonate and ethylene, which cause substantial alterations in gene expression and have complex crosstalk [17,26,35,37]. SA, in addition to being involved in R-gene mediated resistance, is a key regulator of SAR [16,33]. SA-mediated signaling has been best characterized in the *Arabidopsis*, tobacco and cucumber systems, but less is known in plants that have high basal levels of SA such as potato and rice [11,18,30].

Precisely how SAR leads to resistance is not completely understood, but several of the pathogenesis-related genes (PR genes) expressed during the development of resistance are antagonistic to pathogens [1,7,23,38]. Mutants or transgenic plants with impaired SA signaling can show increased susceptibility to pathogens and nematodes [3,6,19]. Mutants with high constitutive levels of SA or that overexpress PR genes, often have enhanced disease resistance [5,6,8], although there may be associated fitness costs [4].

Potato has notable differences in SA-mediated defense signaling from tobacco and *Arabidopsis* including basal SA concentrations of 5–10 μg/g fresh weight that are about 100-fold higher than the levels found in *Arabidopsis* or tobacco [10,42]. After challenge with some pathogens, SA levels increase both in tissue proximal and distal to the infection [25,31]. This increase in SA mediates the development of SAR and causes substantial changes in gene expression including in genes encoding pathogenesis-related proteins.

*Abbreviations:* SAR, systemic acquired resistance; SA, salicylic acid; BTH, benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester; ACN, acetonitrile.

* Corresponding author. Tel.: +1 509 786 9261; fax: +1 509 786 9277.
*E-mail address:* rnavarre@prosser.wsu.edu (D.A. Navarre).

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This raises interesting questions about how SA-mediated signaling operates in plants with high constitutive SA levels and how SAR is triggered. Whether such plants are fully capable of responding to an increase in the already high levels of SA or if the high basal SA levels make cells less competent to perceive or transduce the SA signal as happens for other plant hormones is not clear [40]. Based primarily on the finding that SA did not induce resistance to *Phytophthora infestans* but arachidonic acid did, it was suggested that potato might have poor SA signal perception [43]. Some *Arabidopsis* mutants with high basal levels of SA constitutively express SAR [5] and it has been suggested that high SA levels in potato make it more resistant to disease [39].

We are interested in further characterizing SA-mediated signaling in potato and determined that potato has multiple differences from the model SAR systems. In spite of high basal SA levels, potato was still responsive to SA, although in some conditions potato appeared less responsive to SA or SA functional analogues.

## 2. Materials and methods

### 2.1. Supplies and growth conditions

Plants (*Solanum tuberosum*) were grown in potting soil (Sunshine #1 mix, Sungro) in 7 in. pots in a Conviron CMP4030 growth chamber and fertilized once with approximately 15 g of Osmocote 19-6-12 time-released formula. Plants were grown in a 16 h light period at 22 °C during light and 18 °C during the dark period. The Conviron was programmed so that light intensity peaked at noon and was less early morning and evenings. Cultivars used in this study were Russet Burbank and Russet Norkotah. BTH (benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester) was gift from Syngenta and supplied as a 50% formulation; and harpin was a component of Messenger, a gift from Eden Bioscience. HPLC grade methanol and acetonitrile were purchased from Fisher. The USDA-Agricultural Research Service does not endorse any products.

### 2.2. Determination of salicylic acid

Salicylic acid extraction was based on the method of Gaffney et al. [16] with modifications to allow for a higher throughput approach and recovery. Plant material was ground to a fine powder in liquid nitrogen. Results are the average of three independent extractions. SAG was measured after converting to free SA by acid hydrolysis. Samples were analyzed using an Agilent 1100 with DAD and FLD detection. An Agilent XDB C18 column was used with an isocratic mobile phase comprised of 20% methanol, 5% ACN and 75% 20 mM sodium citrate pH 3.75, at a flow rate of 0.75 mL/min at 35 °C. Recovery rates were determined using *o*-anisic acid as an internal standard and were typically greater than 60%.

### 2.3. Northern analysis

RNA blot analysis was conducted following standard procedures with modifications previously described [28,34]. Total RNA was extracted from leaf discs or whole leaves using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen). Potato PR induction was monitored by Northern blot analysis, using a *PR-1* probe (accession number AJ250136) labeled by random priming and 10 μg total RNA was loaded per lane.

### 2.4. SA loading and leaf disc treatments

Stems were detached with a razor blade and incubated for 3 h in a solution of 2 mM SA to load the leaves with SA. Stems were then removed, rinsed with water and incubated up to 48 h longer in water, followed by SA analysis. All leaf disc and tuber treatments were conducted at room temperature under continual fluorescent lighting and with slow rocking. Plants used for these experiments were 4–6 weeks old.

### 2.5. Field grown plants

For the field experiments, the cultivar Russet Burbank was grown on Washington State University's Center for Precision Agriculture Technology Field Laboratory in Prosser, WA. Potatoes were planted in April and were at least 18–24 in. tall when sampled on the dates indicated in Fig. 4. Basal *PR-1* expression and SA concentrations were determined in the leaves of these untreated plants. Because potato can be quite variable in its basal *PR-1* and SA levels, each sample contained leaves bulked together from three to five different plants. This was done in triplicate for each time point. Thus, three triplicate samples comprising a single time point consisted of leaves from 9 to 15 different plants (Fig. 4A). Leaves were immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

## 3. Results

### 3.1. Potato tissues have high amounts of salicylic acid

Potato leaves are known to have high basal levels of SA [10]. The high SA levels are not restricted to leaves, as high basal levels of SA were present in all the tissues examined in the potato cultivar Russet Norkotah (Fig. 1). Most of the SA was present in the form of the glucoside, SA 2-*O*-β-D-glucoside (SAG), and stems contained the largest amounts of free SA. Even potato roots and tubers had sizeable quantities of SA that, while lower than found in leaves, were nevertheless higher than those found in

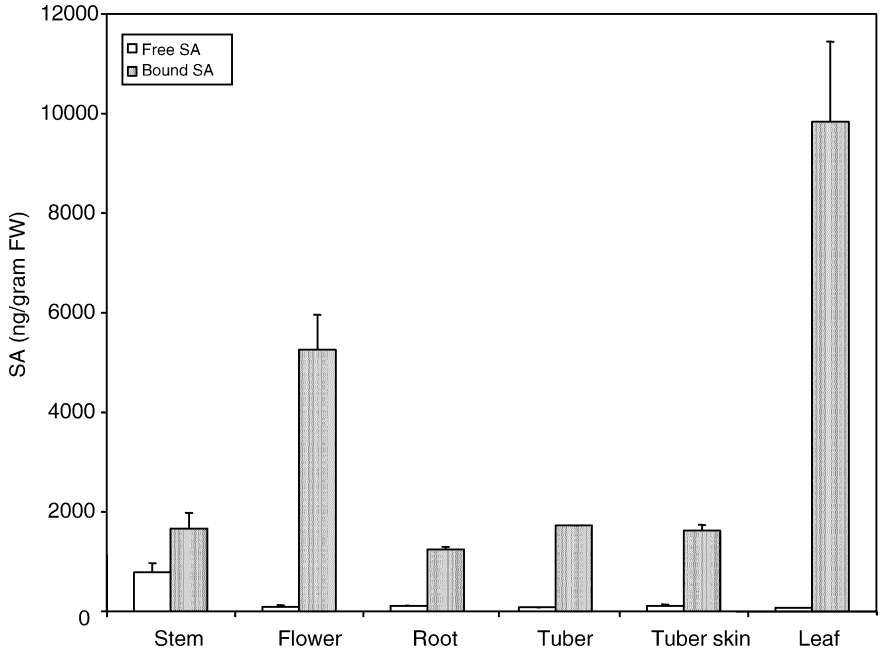


Fig. 1. Salicylic acid concentrations in various Russet Norkotah tissues. SA was extracted from the indicated tissues and measured by HPLC analysis. Leaf samples were from plants 9–10-weeks-old. Results are expressed as nanograms of SA per gram fresh weight tissue. Standard error is shown.

the leaves of many other plants. Both increasing plant age and increasing light intensity caused an increase in SA levels. SA levels in younger leaves from 1-week-old plants grown in lower light conditions (about 25% less than in Fig. 1) were 11.2 ng/g FW (SD ± 3.6 ng) of free SA and 584 ng/g FW (±89) of bound SA, levels markedly lower than found in 9–10-week-old plants grown at higher light intensity (Fig. 1).

### 3.2. Relationship between SA and PR-1 basal expression

In tobacco and *Arabidopsis* little or no basal expression of *PR-1* is present. Potato, however, can have a significant amount of *PR-1* present in leaves in the absence of induction with exogenous compounds (Fig. 2A). Plants were transformed with the bacterial salicylate hydroxylase gene that converts salicylic acid to catechol [16]. As seen in Fig. 2A, *nahG* potato had lower basal expression of *PR-1*, suggesting that the high basal SA level was responsible for the *PR-1* basal expression. *PR-1* was induced when *nahG* plants were treated with 100 μM BTH, but not when treated by 1 mM SA (Fig. 2A, lanes 7 and 8).

In the *nahG* plants, only trace amounts of free salicylic acid were detected. Small amounts of SAG accumulated in *nahG* plants, but large amounts of catechol were produced (Fig. 2B). Despite the large amount of total SA, the potato cultivar Russet Norkotah had the ability to keep free SA levels very low, as did Russet Burbank (data not shown). The wild-type counterparts of the *nahG* plants (Fig. 2B) had 17.8 ng/g FW (±3 ng) of free SA and 3590 ng/g FW (±807 ng) of SAG. Thus, over 99.5% of the total SA was present as SAG. The *nahG* plants all had normal phenotypes until they were about 4–6-weeks-old, at which time they started showing some yellowing and reduced leaf size, effects that increased with age. This was seen in all five independently generated transgenic lines and was considerably more noticeable when plants were grown under higher light intensity. No such effects are seen in tobacco or *Arabidopsis nahG* transgenics.

Elevated basal PR gene expression was noted previously in potato, although the extent of PR expression was variable between experiments and attributed to the plants being stressed [39]. We also observed that basal *PR-1* expression can be quite variable in potato (Fig. 2A, lanes 1–3) but found that growing the plants under optimal light intensity reduced the variation. Such plants had both lower basal *PR-1* expression (untreated samples in Fig. 3A) and lower SA concentrations.

### 3.3. PR-1 expression and SA responsiveness

The high basal levels of SA raise questions about how functional SA-mediated signaling is in potato and it has been suggested that potato might be poorly responsive to SA [43]. Healthy plants, despite having high basal SA levels, were responsive to SA and BTH (Fig. 3A). Spraying plants with as little as 250 μM SA increased *PR-1* expression and 500 μM SA gave a strong induction. However, SA responsiveness can be obfuscated if using plants that have already high basal *PR-1* expression (Fig. 2A, lane 2). Such plants show little or modest additional induction of *PR-1* following SA treatment (data not shown) emphasizing the importance of growing plants in conditions that minimize basal *PR-1* expression.

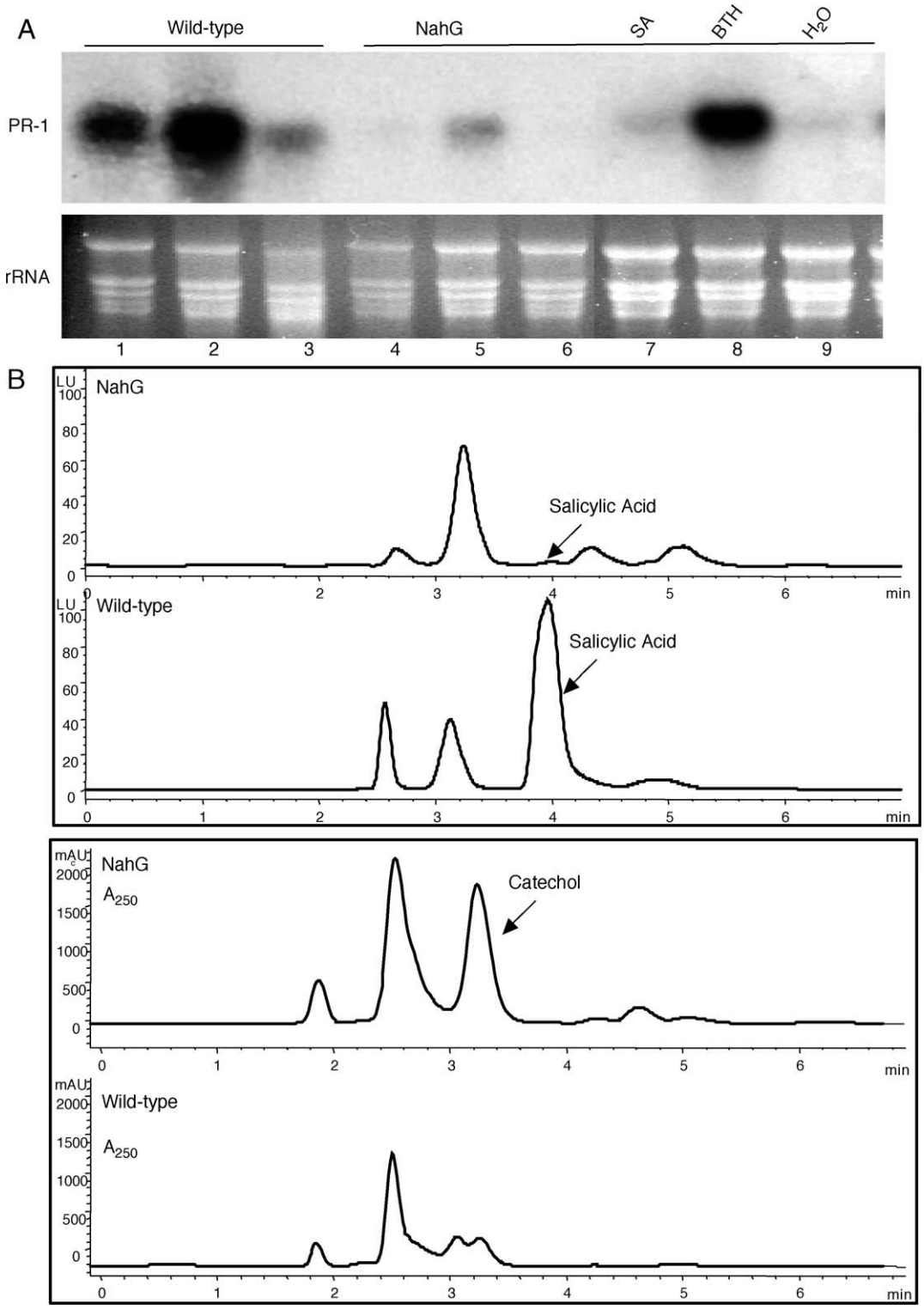


Fig. 2. Northern blots showing *PR-1* expression in leaves from growth chamber-grown, 4-week-old potato plants. (A) Lanes 1–3, basal *PR-1* expression in wild-type leaves, in *nahG* leaves (lanes 4–6), 24 h after treatment of *nahG* plants with 1 mM SA (lane 7), 100 μM BTH (lane 8) or water (lane 9). (B) HPLC chromatographs of the bound SA fraction from wild-type or *nahG* plants showing either FLD or UV detection of the same extract.

The capacity of potato leaves to convert free SA to SAG was evaluated using detached potato stems incubated in 2 mM SA for 3 h. Stems were then removed and placed in water for various times after which free SA and SAG were measured (Fig. 3B). After 3 h SAG increased six-fold (~14,000 vs. 2300 ng) and free SA levels three-fold in the leaves of SA fed plants relative to mock-treated plants. Within 48 h, free SA levels in the SA-fed plant decreased to

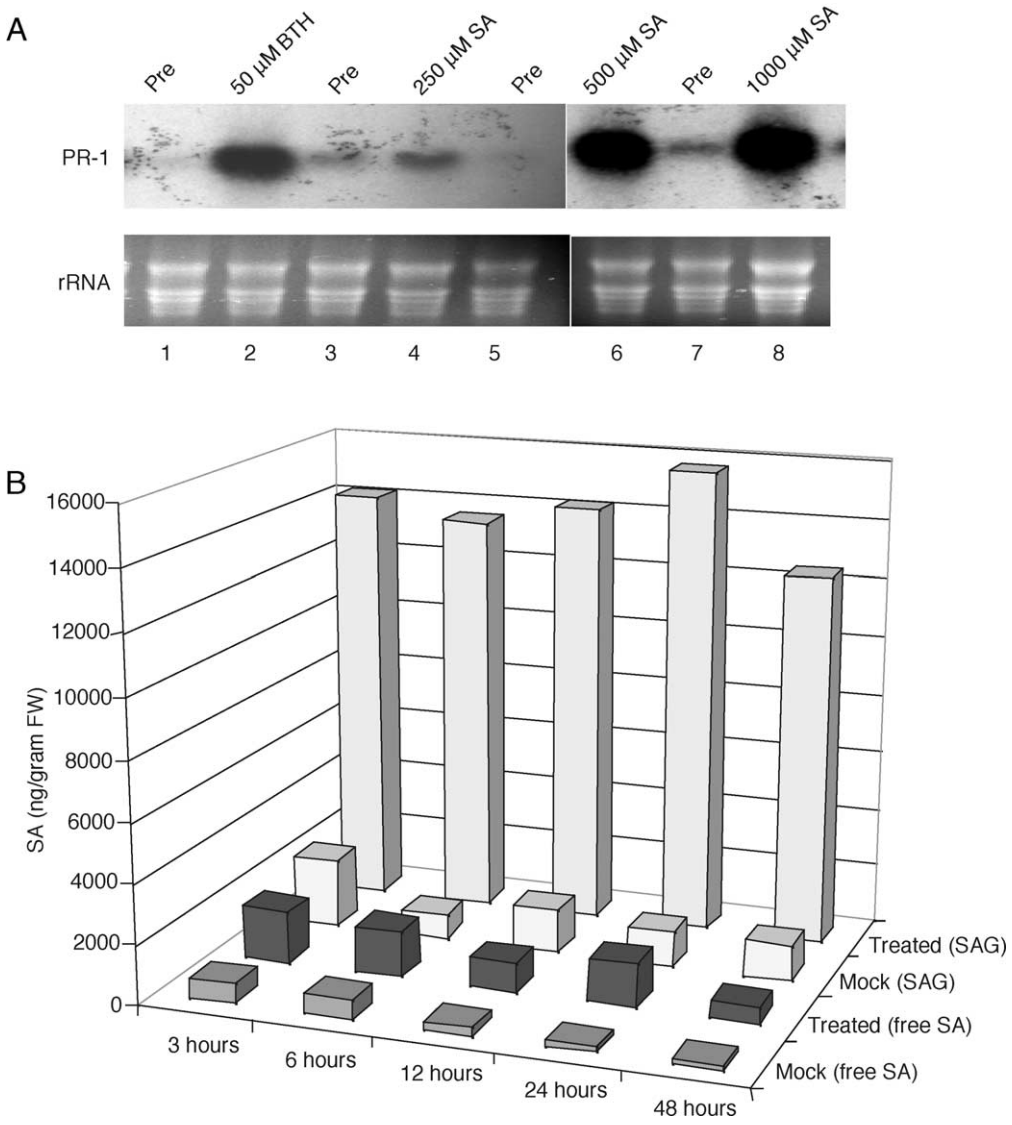


Fig. 3. (A) Northern blot showing *PR-1* expression in leaves of whole plants receiving the indicated treatments. Basal *PR-1* levels from the exact same plant before or 24 h after the indicated treatment. Before or after 50 μM BTH (lanes 1 and 2), 250 μM SA (lanes 3 and 4), 500 μM SA (lanes 5 and 6), or 1 mM SA (lanes 7 and 8). (B) Free and bound SA concentrations in potato leaves preincubated in 2 mM SA. Detached stems were incubated in 2 mM SA for 3 h, and then removed and placed in water. SA levels were then measured at the indicated time points, starting 3 h after removal from the SA solution. For a control, the SA levels are shown in leaves identically treated except pretreatment was in water, not SA.

the basal SA level initially shown by the mock-treated plants, whereas SAG levels were over 10-fold higher (~12,500 vs. 1200 ng) than the SAG concentration in corresponding untreated plants. These data demonstrate that potato is able to keep free SA concentrations low, even while accumulating large amounts of SAG.

### 3.4. PR-1 expression in field conditions

Most studies of SA signaling use plants grown in carefully controlled, pest-free conditions in growth chambers or greenhouses. Consequently, little is known about how the variable environmental conditions encountered in nature impact defense signaling. *PR-1* expression was monitored in potato leaves sampled from the same field over the course of several months. Because potato can be variable in its *PR-1* expression, triplicate samples were collected and each sample was comprised of bulked leaves from three to five different plants (i.e. 9–15 plants per time point). Fig. 4A shows that *PR-1* expression was much lower earlier in the summer and then increased throughout the summer. All sampled plants appeared healthy, with no obvious signs of disease. Levels of free SA did not vary by more than 15 ng/g FW throughout the course of the summer (Fig. 4D). SAG levels were lowest at the earliest time point and increased by 34–48% at later time points. Total SA was lowest at the first time point, which also had the lowest level of *PR-1* expression. However, it was not clear that the later rise in total SA correlates with the increased *PR-1* expression because the July 10th time point had an increase

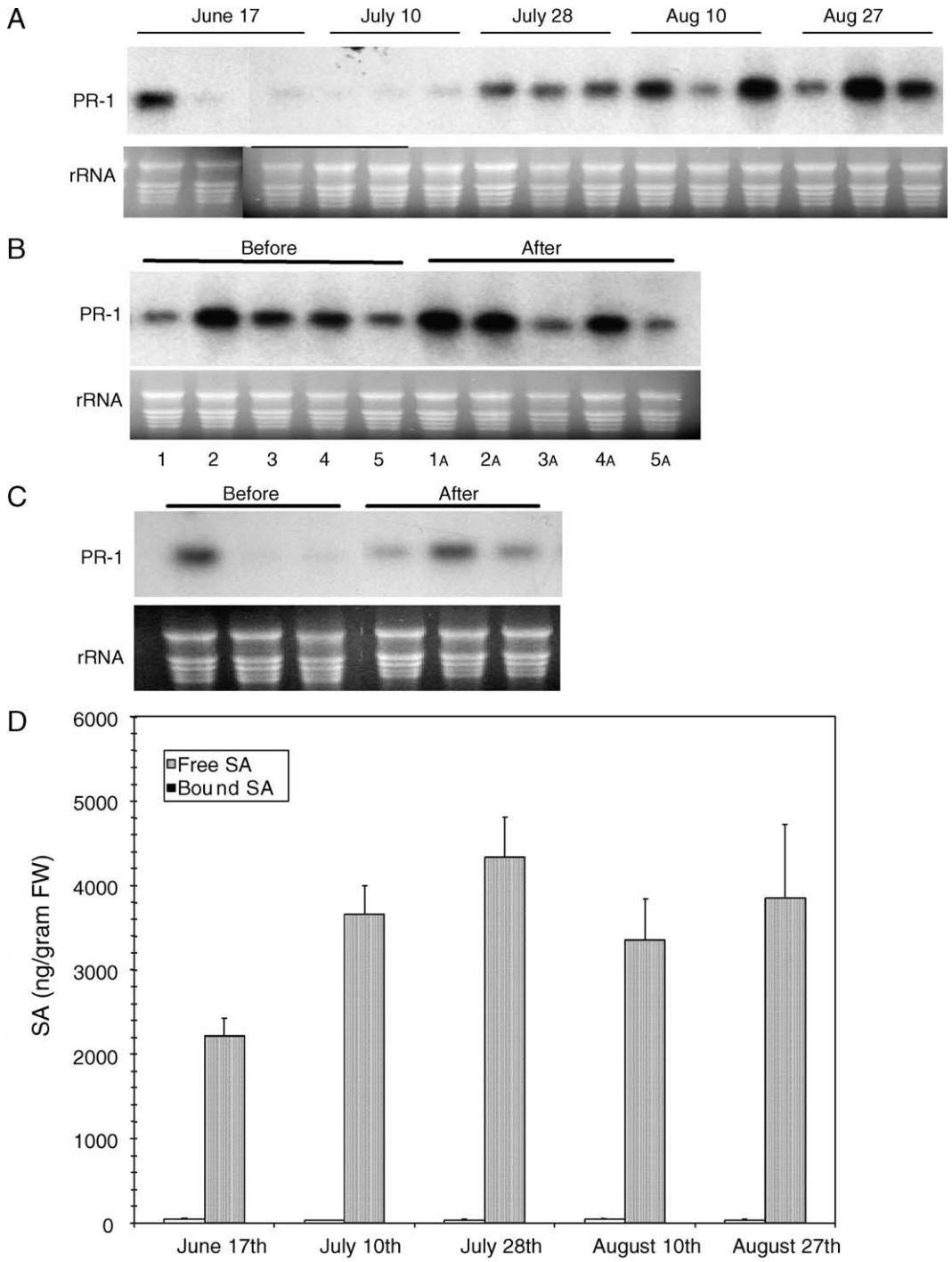


Fig. 4. (A) Basal *PR-1* expression in leaves of untreated potato plants grown in the field. Northern blot shows triplicate samples from each of the indicated time points. (B) *PR-1* expression in field grown plants from the August 27th time point before and 24 h after treatment with 100 μM BTH. Leaves were sampled from the exact same plant before and after treatment. Thus, $1_A$ corresponds to 1, etc. (C) *PR-1* expression in field grown plants from the July 10th time point before and 24 h after treatment with 100 μM BTH. Unlike (C), these do not compare the exact same plant, but rather plants from the same plot before and after spraying. (D) Free and bound SA concentrations in the same plants used in (A). Standard error is shown.

in total SA roughly equivalent to latter time points, but without any increase in *PR-1* expression (Fig. 4A).

Because the plants of the August 27th time point had high basal *PR-1* expression, we assessed how responsive these plants were to further *PR-1* induction. *PR-1* expression was measured in the leaves from five different plants immediately before and 24 h after spraying the exact same plants with 100 μM BTH (Fig. 4B). Curiously, in only one of the five samples (Fig. 4B, compare lanes 1 with 1A) did spraying with BTH result in a large increase in *PR-1* expression. A similar experiment was conducted with the July 10th plants that had low basal *PR-1* expression.

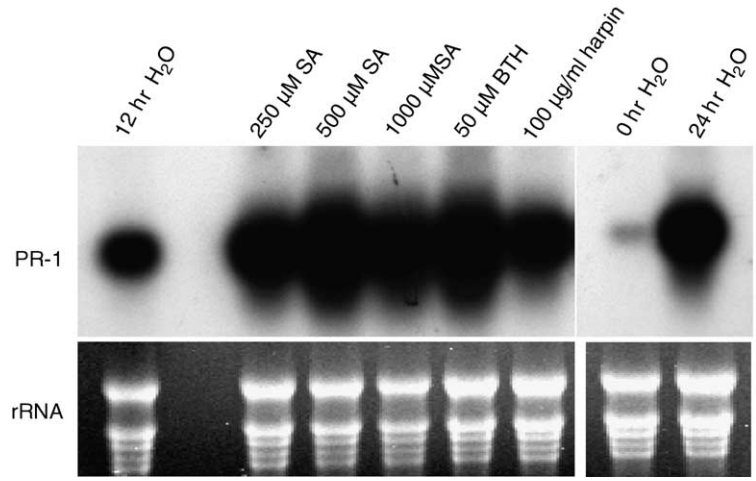


Fig. 5. Northern blot showing *PR-1* expression in treated and untreated potato leaf discs. Leaf discs were incubated in water for 0, 12 or 24 h, or in the indicated treatment for 12 h. rRNA is shown as a loading control.

Treated plants showed only a modest increase in *PR-1* expression (Fig. 4C). This lack of responsiveness was not likely due to *PR-1* already being expressed at maximal levels prior to treatment because the basal levels seen in Fig. 4B (lanes 1, 3–5) and Fig. 4C (July 10th time point) are significantly below the levels usually induced by 1 mM SA or 100 µM BTH in growth chamber grown plants (Fig. 3A). Signal intensities in these figures can be directly compared because the Northern blots shown in Figs. 3 and 4 were hybridized and washed together in the same tube and exposed for the same time.

### 3.5. PR-1 inducibility in leaf discs

Tobacco leaf discs are often used to monitor PR gene expression, but the use of potato leaf discs is problematical. Basal *PR-1* levels were consistently higher in leaf discs than they were in whole plants. Surprisingly, leaf discs incubated in only water had substantially increased *PR-1* expression after 12 and 24 h. *PR-1* was induced above the control levels in leaf discs incubated for 12 h with various treatments including BTH and harpin, however, *PR-1* expression was also high in the untreated 12 h control (Fig. 5). Furthermore, if expression was measured 24 h after various treatments there was no difference in *PR-1* expression in treated vs. untreated samples. This illustrates that the use of potato leaf discs for defense signal transduction studies should be carefully considered, particularly if incubated more than 12 h.

### 3.6. Potato leaf discs are hypersensitive to BTH

When potato leaf discs were incubated with 100 µM BTH they started to disintegrate within 24–48 h. Interestingly, this effect occurred even with BTH concentrations as low as 1 µM (Fig. 6). This is markedly different from tobacco leaf discs, in which concentrations as high as 500 µM BTH had no visible effect. The BTH effect was restricted to leaf discs as no such effect occurred with tuber discs, nor did heavy spraying of whole leaves with 250 µM BTH cause visible damage to leaves. Likewise, incubation of detached leaves in 250 µM BTH had no visible effect. This indicates that wounding is required for the response and that there is a difference that allows

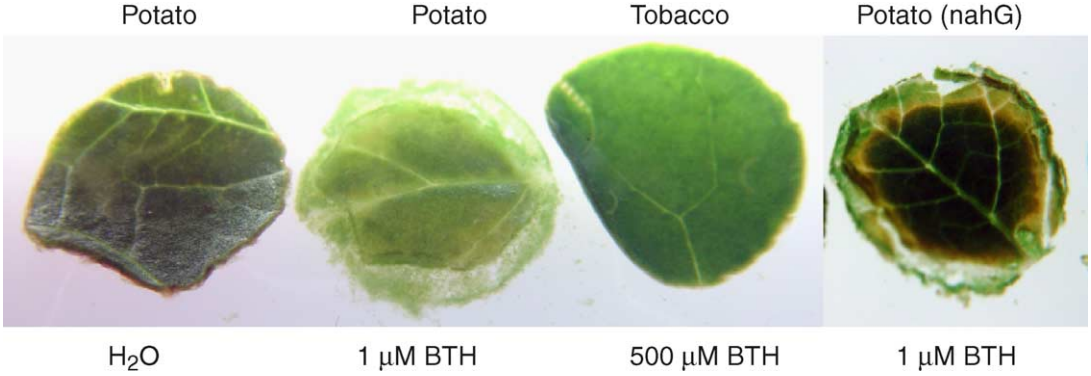


Fig. 6. Potato leaf discs incubated for 48 h in either water or 1 µM BTH, a tobacco leaf disc incubated for 48 h in 500 µM BTH and leaf discs from *nahG* potato 48 h after incubation in 1 µM BTH.

leaves but not tubers to undergo this response. Similar effects were seen with other inducers of SAR (data not shown). A possible cause for this effect might be the high basal SA concentration, which could potentiate potato to respond strongly to BTH. However, *nahG* plants exhibited the same phenotype in response to BTH (Fig. 6), strongly suggesting that the BTH sensitivity was not a consequence of the high SA levels.

## 4. Discussion

The degree to which SA-mediated signaling differs amongst plant species is not well defined. We found potato has notable differences in SA-signaling, suggesting caution should be used before assuming the mechanisms described in the better-characterized *Arabidopsis* and tobacco systems are exactly paralleled in potato. These differences include high basal SA concentrations, elevated basal PR gene expression, yellowing and stunting of older *nahG* plants, high *PR-1* expression in untreated leaf discs and a hypersensitivity to BTH. The yellowing phenotype in *nahG* plants may result because of the large pool of salicylic acid in these plants being metabolized into a correspondingly large amount of catechol. *Solanacea* may have increased sensitivity to catechol, as a similar effect has been seen in tomato [3].

Potato had much greater variability in PR gene expression and SA levels than we find in similar experiments with tobacco or *Arabidopsis*, and this variability complicates any attempt to relate the degree of basal PR gene expression to a cultivar's disease resistance. Others have also noted variability with potato [21,39]. We observed that as light intensity increases so do SA and *PR-1* levels. Potato seems to have the ability to readily express PR genes at high levels in the absence of induction with exogenous compounds. The high basal SA levels could play a role in this, perhaps by resulting in plants that are potentiated and thus more able to trigger defense responses [9,20,36] or perhaps the large pool of SAG is readily converted to free SA in response to subtle, as yet undefined stimuli. Much lower basal *PR-1* expression was observed in *nahG* plants, confirming that the high constitutive SA concentrations are a factor in basal *PR-1* expression in potato (Fig. 2). Trace amounts of *PR-1* were occasionally observed in *nahG* plants, which might suggest a minor contribution from either SA-independent factors or elements downstream of SA in the signaling pathway (Fig. 2).

The high basal SA concentration raises questions about whether potato is responsive to SA. Yu et al. [43] suggested potato might have poor SA perception. We found that potato grown under optimal conditions is capable of expressing *PR-1* in response to even low concentrations of SA (Fig. 3A). This suggests that although potato has high amounts of total SA, it is capable of tightly regulating the amount of free SA, and this may allow tissue to remain responsive to free SA. In the cultivars Russet Burbank and Russet Norkotah over 99.5% of the total SA was bound, and free levels were comparable to levels found in tobacco. However, at other times field-grown potato seemed less responsive to inducers of SAR (Fig. 4B and C) suggesting that potato may have a differential competence in its ability to perceive or transduce the SA signal. A possible explanation for the reduced responsiveness might be if jasmonate levels are elevated in field grown plants, because jasmonate can be antagonistic to SA-mediated signaling [29]. Thus, conditions such as insect or drought pressure in the field might impair SA-mediated signaling.

In many ways, potato more closely resembles various mutant, rather than wild-type *Arabidopsis*. The SA levels in potato are roughly equivalent to the elevated total SA levels found in the *Arabidopsis lsd6* and *lsd7* mutants that exhibit spontaneous lesion formation [41]. Interestingly, potato grown indoors is prone to spontaneous lesion formation called oedema, which is characterized by the appearance of small callus-like warts on the leaves that become necrotic lesions [27]. The ability to accumulate large amounts of SA is necessary for occurrence of runaway cell death in some *Arabidopsis* mutants and a SA signal potentiation loop was proposed [2]. Exogenous SA can cause runaway cell death in *lsd1* mutants, but not wild-type *Arabidopsis* [12].

The extensive cell death in leaf discs caused by BTH was unexpected. Once again, this is a potato trait more in common with some *Arabidopsis* mutants than wild-type plants. BTH doses as low as 10 μM hyperactivate cell death in *acd6* mutants, and BTH also caused cell death in plants overexpressing *ACD6* [24,32]. *Arabidopsis* overexpressing *NPR1* also show enhanced responsiveness to BTH [15]. Rice, like potato, has high basal levels of SA, and *NPR1* overexpressed in rice, but not *Arabidopsis*, can trigger a lesion mimic/cell death phenotype [13]. We thought the effect of BTH in potato might be due to the high SA levels that perhaps potentiated a strong response to BTH. However, BTH treatments induced the same phenotype in *nahG* plants, showing that while the SA-mediated signaling pathway is involved, the high basal levels are not the reason potato exhibits this phenotype. BTH acts downstream of SA in the SA-mediated signaling pathway [14] and this can be seen in Fig. 2 (lane 8) where BTH induces PR1 in *nahG* plants. Because neither wounding nor BTH alone caused this phenotype, both wounding and SA-mediated signaling are required. Efforts to identify the mechanism of this BTH-induced cell death are underway.

*PR-1* expression increased throughout the growing season in plants grown outdoors (Fig. 4A). One explanation for this would be if the plants faced increased disease pressure as the season progressed and responded by activating defense genes. However, these plants had no obvious visible symptoms to suggest they might be diseased nor was it clear that an increase in SA concentration was responsible (Fig. 4D). Whether increased PR gene

expression as the growing season progresses is a common occurrence in plants is a question that has implications for chemically induced SAR disease control strategies, as does the apparently variable responsiveness of potato to compounds that induce SAR. To date, SAR has not been successfully adopted for disease control in potato nor in many other crops. However, with the rapidly expanding characterization of SA-mediated signaling in plants, SAR may become an increasingly viable strategy for disease control.

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